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FULL PAPER

Restriction Factors expression decreases in HIV-1 patients after cART

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SUMMARY

Activation of interferon (IFN) mediated responses and the consequent expression of restriction factors (RFs) represent an early line of defense against HIV-1 infection. The levels of viral replication and the antiviral are among the determinants influencing RFs' expression pattern. A deeper understanding of the molecular mechanisms regulating RFs activity and their relationship with viral replication factors might lead to new therapeutic strategies based on the enhancement of immune response against the virus. The aim of this study is to perform a longitudinal evaluation of the variations in the levels of a group of selected RFs (APOBEC3G, BST2, TRIM5α, MX2, SAMHD1, SERINC3/5, IFI16 and STING) to determine the impact of cART on their expression in HIV-1 positive patients.

Together with RFs expression, immunological and virological parameters (plasma HIV1-RNA load and total HIV1-DNA) were longitudinally evaluated in a cohorts fourteen HIV-1 cART naïve patients, who were evaluated at diagnosis (T0) and followed at 4 (T1) and 8 (T2) months after starting cART. Fourteen long-term treated patients who achieved sustained undetectable viremia for at least 2 years were also included in the study as a reference group.

We observed a restoration of immunological conditions during cART, together with a progressive decrease of HIV1-RNA load, which became undetectable at 8 months after starting treatment. On the other hand, despite showing a trend towards decrease, total HIV1-DNA remained detectable after reaching viral suppression, similarly to what observed in long term treated patients.

The expression of APOBEC3G, SAMHD1, BST2, IFI16, SERINC3, and SERINC5 was higher at the time of diagnosis and decreased significantly during therapy, reaching levels similar to the ones observed in virally suppressed patients. On the other hand, MX2 and TRIM5a high expression values up to T0, reaching lower levels immediately after the initiation of cART treatment.

Correlation analysis showed a positive association between the expression levels of APOBEC3G, IFI16, MX2, SAMHD1, SERINC3 and TRIM5α with the HIV-1 viral load. On the contrary, no significant association was observed for BST2, SERINC5 and STING, even BST2 expression showed a tendency to correlate with viral load. We observed a tendency for a positive association of MX2, SAMHD1 and SERINC5 with the size of viral reservoir and a trend for a negative association for STING. STING appeared also as the only one factor whose expression correlates with the CD4 count and the CD4/CD8 ratio.

Our data confirm the correlation between viral replication and expression of RFs, with, the levels of cellular defense proteins decreasing in parallel to the reduction of viral replication.

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INTRODUCTION

HIV-1 infection is one of the most relevant health problems worldwide and more than 36 million people are currently living with HIV-1. Despite the introduction of combined antiretroviral therapy (cART),

which is able to maintain the viral load below undetectable values, HIV-1 persistence represents a major obstacle to viral eradication (Howgego GD., 2021).
Although several studies have described the complex landscape of host-viral interaction during the infection (Bösl *et al.*, 2019; Jones et al., 2020; Kachuri *et al.*, 2020), many aspects of the viral pathogenic mechanisms and host response still require investigation and analysis. Among the growing body of studies focusing on the pivotal role of the immune system in modulating pathogenesis of HIV infection, several analyses have pointed out the role of cellular defense proteins in the control of viral replication. These proteins, collectively known as restriction factors (RFs), are able to limit viral replication by acting on essential steps of the viral cycle, including uncoating, reverse transcription, nuclear import-integration and budding (Malim and Bieniasz, 2012; Romani and Allahbakhshi, 2017). Because of their ability to respond to interferon (IFN) stimulation, RFs are considered a branch of the interferon-induced molecular reactions. HIV-1 related chronic inflammation exerts a continuous stimulus on IFN induction, in particular for type I and II IFNs, increasing the expression of interferon stimulated genes (ISGs), including RFs. Of note, in the complex scenario of drivers determining HIV-1 persistence, it has been proposed that expression of RFs might play a role in the onset of latency (Abdel-Mohsen *et al.*, 2015; Merindol and Berthoux, 2015).
The most characterized RFs for HIV-1 are apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G), Tetherin (BST-2), TRIM5α/11, MX2, sterile alpha motif histidine-aspartic (SAMHD1), and SERINC3/5 (D'Urbano *et al.*, 2018). This RFs are generally classified based on the step of viral cycle that they target: SERINC3/5 prevent fusion and uncoating of viral particles (Rosa *et al.*, 2015; Usami *et al.*, 2015), TRIM5α, APOBEC3G and SAMHD1 hamper reverse transcription (Yuan *et al.*, 2016; Morger *et al.*, 2017, Fu *et al.*, 2016; Malim and Bieniasz, 2012; Holmes *et al.*, 2015), MX2 interferes with translocation of viral genome through the nuclear membrane (Dicks *et al.*, 2015), and BST-2 limits the spread of infection by tethering the virions on the cellular surface (Dicks *et al.*, 2015).
Several studies have described RFs upregulation in HIV-1 positive patients during the different phases of infection, suggesting that RFs expression patterns reflects the variation in virus replication and that, in turn, RF expression might influence key aspects of HIV-1 pathogenesis (de Pasquale *et al.*, 2013; De Masson *et al.*, 2014; Malatinkova *et al.*, 2015). In this study, we examined the expression levels of a restricted panel of RFs in a group of HIV-1 infected individuals at first diagnosis and after starting antiretroviral treatment in order to analyze the relationships between gene expression variations and the viral reservoir.

MATERIALS AND METHODS

HIV-1 patients and controls

We enrolled 28 Caucasian subjects infected with HIV-1 (group B). Group 1 [mean age 44 (35-47)] included fourteen newly diagnosed HIV-1 infected subjects with long-lasting infection (estimated conversion time exceeding six months at the time of diagnosis established by avidity tests) (Musumeci et al., 2016; Suligoi et al 2011). Group 2 [(mean age 51 (43-60)] comprised fourteen HIV-1 treated patients on stable cART with viral load undetectable for at least 2 years. Blood samples were collected at the time of HIV-1 diagnosis before starting antiretroviral treatment (T0) and after 4 (T1) and 8 (T2) months from the beginning of cART from patients belonging to group 1, while for patients included in group 2 a single blood sample was drown during one of the routine control visits.
The study protocol was evaluated and approved by the local Ethical Committees of Area Vasta Emilia Centro of Emilia-Romagna (CE-AVEC, protocol number 489/2018/Sper/AOUBo), all patients provided written informed consent before being enrolled in the study.

Analysis of immune status and viral replication

CD4+ and CD8+ T cell count and CD4/CD8 ratio were determined by flow cytometry (FACScan, Becton & Dickinson, Mountain View, CA, USA) using commercially available monoclonal antibodies, as previously described (Perfetto and McCrary, 1999).
Plasma HIV-RNA load was determined using a commercial viral RNA detection assay (COBAS® AMPLICOR, Roche Molecular Systems, Inc., Branchburg, NJ, USA) with a lower limit of detection of 20 HIV-RNA copies/mL (LoD by hit rate of ≥95%). Samples giving no signal at PCR or for which Ct values were below the limit for the assay were reported as "HIV-1 RNA not detected" (TND).
Total HIV-1 DNA was quantified using a diagnostic kit (Diatheva, Italy). DNA was extracted from PBMCs using Quick DNA™ Microprep Kit (Zymo Research Corp.), and eluted in 50 µl of nuclease free water. Concentration of DNA was determined using NanoDrop2000 spectrophotometer (ThermoFisher Scientific). Following the manufacturer's instruction, 1µg of purified DNA (corresponding approximately to 1.5 x10^5 PBMCs) was used for this analysis in 50 µl of reaction mix. The reaction was performed on the Versant kPCR Molecular System (Siemens) with the following cycling conditions: 95°C for 3 minutes, 50 amplification cycles of 95°C for 20 seconds, 60°C for 60 seconds. The results were expressed as HIV1-DNA copy number/10^6 PBMCs.

Restriction factors analysis

PBMCs from HIV-1 positive patients and healthy donors were isolated from fresh whole blood using

Ficoll density gradient centrifugation (Ficoll-Paque PLUS Pharmacia, Uppsala, Sweden) and stored at -80°C for subsequent nucleic acids extraction.

Total cellular RNA was extracted using the QuickRNA™ Miniprep Kit (Zymo Research Corp.), according to the manufacturer's instructions. DNase treatment was performed to prevent possible genomic contamination. Total RNA was eluted in 50 µl of nuclease free water and stored at -80°C until molecular analysis.

RNA concentration was measured using NanoDrop2000 r and 500 ng was reverse transcribed into cDNA using cDNA Synthesis Kit (Biotechrabbit™) according to the manufacturer's instructions. A negative control of reverse transcription was included for each primer pair to exclude non-transcribed genomic DNA contamination.

cDNA was diluted to a final concentration of 5 ng/µl and 25 ng were used in a 20 µl real time PCR reaction mix. The reaction mix includes 10 µl of 2X buffer (QuantiFast SYBR Green PCR kit, Primerdesign) and 0.3 µM of primer-mix. Real time PCR were performed on Rotor-Gene Q (Qiagen) under the following conditions: 95°C for 10 minutes, 40 amplification cycles of 95°C for 15 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The melting curve for assay specificity was set up from 60°C to 95°C. RFs primers were designed using SnapGene Viewer 4.1.8 and were tested for the specificity using BLAST program. Primer sequences are listed in *Table 1*.

Relative gene expression values of RFs were calculated according the 2ΔΔCt method, using β-actin (*ACTB*) gene for normalization (Khurshid *et al*., 2009; Mukai *et al*., 2009; Van Hecke *et al*., 2019; Zhao *et al.*, 2016). The average TND Ct values were used as reference to calculate fold variation.

Statistical Analysis

Data were analyzed using GraphPad PRISM 5 software. The multiple group comparisons were assessed using Kruskal-Wallis test followed by Dunn's correction. The threshold for statistical significance was set to 0.05. To evaluate and visualize the relationship between the RFs expression levels and clinical parameters, we performed Spearman rank-based correlation using the corrplot package from R Bioconductor (R version 4.0.3) (Wei *et al*., 2017). R package corrplot: Visualization of a Correlation Matrix (Version 0.84)].

RESULTS

Clinical characterization of the patient cohorts

Table 2 summarizes the immunological and virological parameters of HIV-1 positive individuals (group 1 and group 2). Patients belonging to group 1 were evaluated for 8 months after beginning of therapy: the average CD4 + T lymphocytes count was 235.5 cell/mmc (IQR=94.5- 436.5) at baseline, with a statistically significant increase at T1 (406.5 cell/mmc, IQR=289.5-629.5; p<0.001) and T2 (489.5 cell/mmc, IQR=324.3-726.3; p<0.001) respectively.

We did not observe significant differences in CD8+ T lymphocytes percentage after start of therapy [60.5% (IQR=51.50-70) at T0, 54.5% (IQR=49.75-69.25) at T1 and 54% (IQR=48.7-65.5) at T2. Similarly, CD4/CD8 ratio did not change in response to treatment, with average values 0.275 (IQR=0.125-0.465), 0.345

Table 1 - *Sequence primers pairs for RF used in Real Time PCR. FP, forward primer; RP, reverse primer.*

GENE NAME	*GenBank ID*	*PRIMER SEQUENCE*	*Position cDNA*
APOBEC3G	NM_021822	FP: 5'-TAGCCGGCCAAGGATGAAGC-3' RP: 5'-TCCGACGAGAAAGGATGGGTC-3'	345-364 428-448
MX2	NM_002463	FP: 5'-AACACCGAGCTAGAGCTTCAGG-3' RP: 5'-AGGTCAATGATGGTCAGGTCTGGAAC-3'	627-648 756-781
SAMHD1	NM_015474	FP: 5'-AGGTGTGCTCCTTCCTCAGG-3' RP: 5'-AAGGCAGTAATGCGCCTGTG-3'	346-365 419-438
SERINC3	NM_006811	FP: 5'-ACCACCGTGTTAGAAAGCAGC-3' RP: 5'-AGCAACTACACAGCAAACATGAGG-3'	111-131 227-250
IFI16	NM_005531	FP: 5'-AAGGAGCAGAGGCAACTCCTG-3' RP: 5'-CACCTTACTCCCTTTGGGTCCAG-3'	643-663 700-722
BST2	NM_004335	FP: 5'-CAGAAGGGCTTTCAGGATGTGG-3' RP: 5'-TTGTCCTTGGGCCTTCTCTGC-3'	313-334 391-411
SERINC5	NM_001174071	FP: 5'-AGCACCCGCTTCATGTACG-3' RP: 5'-CAGGTGTCACCAGCTTTAATGCC-3'	260-278 383-405
STING	NM_001301738	FP: 5'-TGAACATCCTCCTGGGCCTCAAGG-3' RP: 5'-GGATCAGCCGCAGATATCCGATG-3'	482-505 585-607
TRIM5α	NM_033034	FP: 5'-GGGCAGAAGTAGGAAGTCTTTGGG RP: 5'-AGGAGTTCCAGGCAGATGGG-3'	199-222 349-368
TRIM11	NM_145214	FP: 5'-CCGGAAGCAGATGCAGGATG-3' RP: 5'-TCGAACTCACCCAGCACGTTC-3'	693-712 783-803
ACTB	NM_001101	FP: 5'-GAC AGG ATG CAG AAG GAG ATT ACT-3' RP: 5'-TGA TCC ACA TCT GCT GGA AGG T-3'	1015-1038 1135-1156

Table 2 - *Immunological and virologic characteristic of HIV patients (group 1 and 2). Values are reported as median (IQR); T0, blood sample prior to cART; T1, first follow-up and blood sample collection at 4 months after cART; T2, second follow-up and blood sample collection at 8 months after cART; T0, T1, T2 are referred at group 1 individuals. TND, target not detected, patients belonging to group 2, aviremic and under cART for at least 2 years.*

	Group 1			*Group 2*
	T0 (n=14)	*T1 (n=14)*	*T2 (n=14)*	*TND (n=14)*
CD4+ T lymphocytes (cell/mmc)	235.5 (94.75-436.5)	406.5 (289.5-629.5)	489.5 (324.3-726.3)	787 (575.5-1171)
CD8+ T lymphocytes (%) (IQR)	60.5 (51.50-70)	54.5 (49.75-69.25)	54 (48.75-65.5)	40 (29.25-44.25)
CD4/CD8 (IQR)	0.275 (0.125-0.465)	0.345 (0.127-0.592)	0.480 (0.177-0.625)	0.925 (0.69-1.56)
Log VL (cp/ml) (IQR)	4.67 (4.41-5.23)	1.30 (0-1.5)	0 (0-0)	0 (0-0)
DNA Log copies/106PBMCs (IQR)	3,192 (2,901-3,665)	2,496 (1,918-2,967)	2,404 (1,904-2,531)	1,642 (1,544-2,141)

(IQR=0.127-0.592) and 0.480 (IQR=0.177-0.625) at baseline, T1 and T2 respectively.

In patients belonging to group 2, CD4+ T lymphocytes count (787 cell/mmc, IQR=575.5-1171) was significantly higher than in group 1 patients at T0 ($p<0.001$) and T1 ($p<0.05$), but not at T2. Consistently, these patients also showed a lower percentage of CD8+ T lymphocytes (40%, IQR=29.25-44.25) compared to subjects enrolled in group 1 at T0 ($p<0.001$), T1 ($p<0.001$) and T2 ($p<0.01$) as well as a significantly higher CD4/CD8 ratio (0.925, IQR = 0.69-1.56; $p<0.001$).

As expected, HIV-RNA plasma levels were drastically reduced after cART initiation, decreasing significantly from baseline (4.67 log copies/ml, IQR=4.41-5.23) to T1 (1.30 log copies/ml, IQR=0-1.5; $p<0.001$) and T2 (0 log copies/ml, IQR=0-0; $p<0.001$).

The amount of total cellular HIV DNA was higher (3,192 log copies/10^6 PBMCs, IQR=2,901-3,665) at T0 compared to T1 (2,496 log copies/10^6 PBMCs, IQR=1,918-2,967) and T2 (2,404 log copies/10^6 PBMCs, IQR=1,904-2,531). Consistent with the notion of a progressive decay of HIV proviral load during prolonged viral suppression, total cellular HIV DNA levels were significantly lower in group 2 (1,642 log copies/10^6 PBMCs, IQR=1,544-2,141) compared to group 1 at all time-points examined.

RFs expression decreases in HIV-1 patients after cART

The analysis of RFs expression during the first 8 months of cART showed that all RFs, with the exception of STING, trended towards the expression levels observed in group 2 patients.

Overall, we observed a dual impact of cART on RFs expression, with some RFs being immediately downregulated after starting cART, and others being upregulated or stable during the first 4 months of treatment and downregulated following 8 months of therapy.

Compared to TND group, levels of APOBEC3G, SAMHD1, BST2, IFI16, SERINC3, and SERINC5 were significantly higher at both T0 and T1, showing a trend of decrease from T1 to T2 (*Figure 1A-F*). On the other hand, MX2 and TRIM5a expression dropped significantly already at first time-point examined, reaching levels comparable to TND at T1 (*Figure 2A-B*).

Interestingly, STING showed a different trend in its expression, with stable levels at T0 and T1 and a statistically significant overexpression at T2, compared to TND patients (*Figure 2C*).

Correlation between RFs expression and clinical characteristics

Because restriction factors play a pivotal role in viral infection control, particularly at the beginning of viral replication and spread, we evaluated the association between the analyzed RFs with the virologic and immunological parameters. This analysis was restricted to group 1 patients for whom data from multiple time points was available, pooling together all the samples in the analysis (42 PBMCs samples, i.e. 14 samples for each point).

As shown in *Figure 3*, the downregulation of the six restriction factors from baseline (T0) to the end of follow-up (T2), even if with different dynamics, correlated with the decrease of viral load. In particular, a positive correlation between plasma HIV-RNA and RFs expression level was found for APOBEC3G ($p=0.041$, $r=0.316$), IFI16 ($p=0.005$, $r=0.430$), MX2 ($p=0.0007$, $r=0.501$), SAMHD1 ($p=0.005$, $r=0.424$), SERINC3 ($p=0.015$, $r=0.372$), TRIM5α ($p=0.001$, $r=0.485$). On the contrary, no significant association was observed for BST2, SERINC5 and STING, even BST2 expression showed a tendency to correlate with viral load ($p=0.07$, $r=0.282$).

Parallel to the significant decrease of HIV1-DNA during the follow up, we did observe a tendency for a positive association of MX2, SAMHD1 and SER-

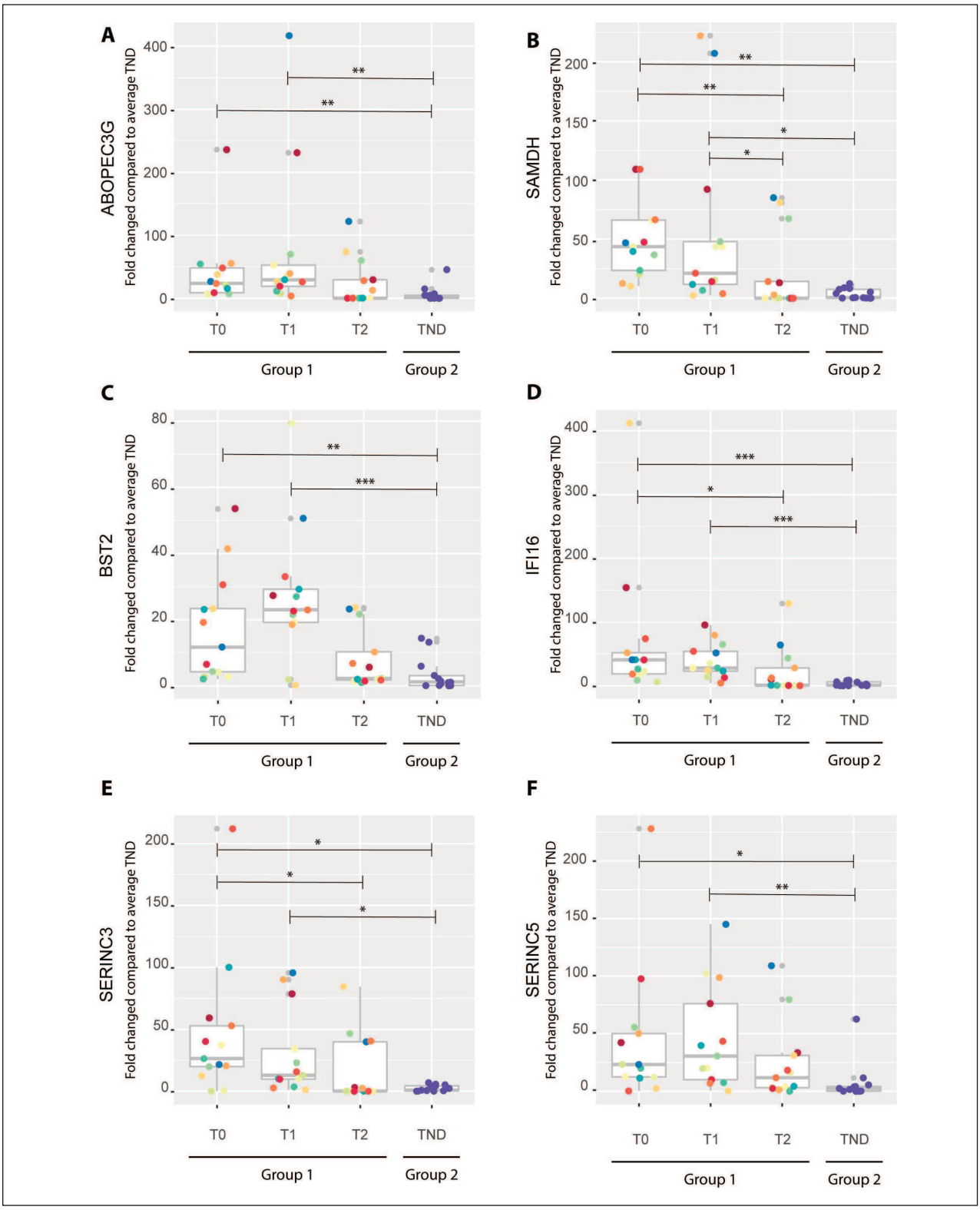

Figure 1 - *Expression profile of APOBEC3G, SAMDH, BST2, IFI16, SERINC3, and SERINC5-*
*Box plot showing the fold change for each RFs in patients included in the study, Group 1 patients were analyzed at before starting therapy (T0) and, subsequently at 4 (T1) and 8 (T2) months of treatment. Group 2 patients (TND) were analyzed once. Fold change was calculated based on the average Ct values of Group 2 patients. For each time point, every dot represents one patient, boxes indicate the interquartile range; for Group 1 each color represents values from the same patient. P values were calculated using the Kruskal-Wallis test with Dunn's correction. Asterisks indicate levels of significance ***p<0.001; **p<0.01; *p<0.05.*

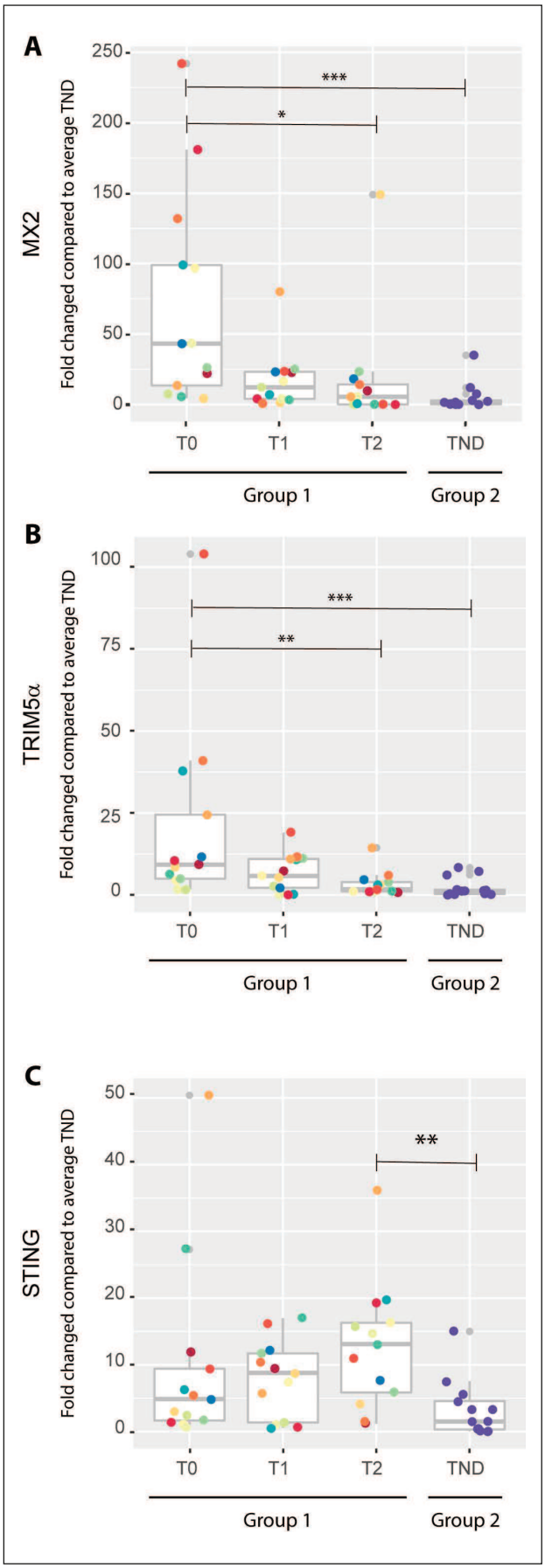

Figure 2 - *Expression profile of MX2, TRIM5, and STING.*
*Box plot showing the fold change for each RFs in patients included in the study, Group 1 patients were analyzed at before starting therapy (T0) and, subsequently at 4 (T1) and 8 (T2) months of treatment. Group 2 patients (TND) were analyzed once. Fold change was calculated based on the average Ct values of Group 2 patients. For each time point, every dot represents one patient, boxes indicate the interquartile range; for Group 1 each color represents values from the same patient. P values were calculated using the Kruskal-Wallis test with Dunn's correction. Asterisks indicate levels of significance ***p<0.001; **p<0.01; *p<0.05.*

INC5 with the size of viral reservoir (p=0.09, r0.286; p=0.07, r= 0.280; p=0.05, r=0.294), and a trend for a negative association for STING (p=0.09, r=-0.260). Interestingly, levels of STING also showed a positive correlation with the CD4+ cell count (p=0.005, r=0.425) and the CD4/CD8 ratio (p=0.029, r= 0.337), and did not correlate with the CD8 percentage (p=0.07, r=-0.274) (*Table 3*).

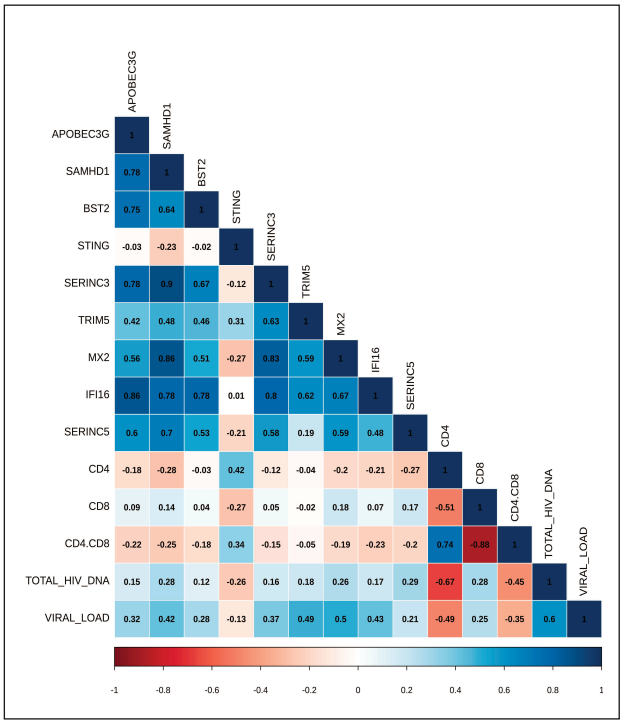

Figure 3 - *Correlation analysis between RF and plasmatic viral load in group 1 patients (42 observations). P and r-values were calculated using Spearman correlation test. All values <0.05 were considered statistically significant.*

Table 3 - *Correlation analysis between the RF expression levels and clinical characteristics of Group 1 patients.*

Spearman correlation analysis	*CD4 / mmc*		*% CD8*		*CD4 / CD8*		*Plasma HIV1-RNA*		*Total HIV1- DNA*	
	P value	*r*	*P value*	*r*	*P value*	*r*	*P value*	*r*	*P value*	*r*
APOBEC3G	0.264	-0.176	0.583	0.087	0.169	-0.216	*0.041*	*0.316*	0.333	0.153
BST2	0.830	-0.034	0.787	0.043	0.258	-0.179	0.07	0.282	0.453	0.119
IFI16	0.189	-0.207	0.653	0.071	0.144	-0.230	*0.005*	*0.430*	0.271	0.174
MX2	0.205	-0.199	0.263	0.177	0.232	-0.188	*0.0007*	*0.501*	0.096	0.260
SAMHD1	0.076	-0.276	0.361	0.144	0.114	-0.247	*0.005*	*0.424*	0.077	0.280
SERINC3	0.455	-0.119	0.741	0.053	0.342	-0.151	*0.015*	*0.372*	0.297	0.165
SERINC5	0.084	-0.270	0.274	0.173	0.202	-0.201	0.188	0.207	0.058	0.294
STING	*0.005*	*0.425*	0.078	-0.274	*0.029*	*0.337*	0.423	-0.127	0.09	-0.260
TRIM5α	0.816	-0.037	0.916	-0.017	0.757	-0.049	*0.001*	*0.485*	0.245	0.183

DISCUSSION

This study investigated the expression levels of RFs during the course of HIV-1 infection in a group of patients enrolled at the moment of first diagnosis, when naïve to treatment, and followed up at 4 and 8 months after starting cART.

Although all RFs showed an overall decrease associated with the reduction of viral replication, RFs dynamics as well as their correlation with virologic and immunological parameters varied depending on the RFs considered.

Our observations are in line with previous studies that reported the association between the expression of specific restriction factors and viral replication in HIV-1 infected patients, likely due to the increased viral antigen and interferon exposure. Using a complex score accounting for the variations of all RFs, Abdel-Mohensen *et al*. (2015) showed that RFs are overexpressed in untreated viremic patients compared to both ART-suppressed and elite controllers. In a study analyzing RFs expression that grouped patients by treatment status, control of viral replication, and time of start of antiretroviral therapy, Van Hecke *et al*. (2019) confirmed that viral load drives the expression of specific RFs.

Interestingly, the same study also showed that levels of RFs were higher in patients who started treatment at seroconversion compared to patients who started treatment during chronic infection, suggesting that early therapy initiation might help in maintaining the functionality of the IFN axes (Ashok kumar *et al.*, 2020; Goujon and Malim, 2010; Lavender *et al.*, 2016). Given that the time from infection event and diagnosis in the patients included in the present study is unknown, we cannot determine whether this observation applies also to our data.

The association of CD4 levels with RFs expression is still debated, with some authors reporting a positive correlation suggesting that cART induced CD4 count recovery pairs with a restoration of innate immune function, and others reporting the lack of significant association. These discordant results could depend on several factors, including study cohort characteristics as well as sampling of total PBMCs vs CD4 T cells or specific CD4+ T cell populations (Abdel-Mohsen *et al.*, 2015; Bachtel *et al.*, 2018; Van Hecke *et al.*, 2019).

Contrary to what reported by Van Hecke et al, our data did not show a significant association between RFs expression and viral reservoir, measured as total HIV1-DNA. This can be the reflection of the different of decay between RNA and DNA viral load. Indeed, although HIV1-DNA declines during cART, it remains quantifiable also after plasma HIV1-RNA levels are below undetectable levels (Bon *et al.*, 2017; Suligoi *et al.*, 2011; Re *et al.*, 2010). This is due the persistence of unintegrated HIV1-DNA forms and defective copies of integrated virus, which have a longer half-life compared to HIV1-RNA. Defective proviruses also account for the production of unspliced HIV-RNA molecules that enhance inflammation and immune activation, supporting a residual activity of the interferon response beyond the achievement of viral suppression (Imamichia *et a*l., 2016). Consistently, in the cohort of TND patients, who experienced long term suppression of viral replication, levels of HIV DNA as well as RFs expression were significantly lower than in group 1 patients.

Strikingly, STING showed a trend opposite to the other RFs, maintaining stable levels, significantly higher compared to TND, over the three time-points considered in the analysis. STING is ubiquitously expressed in various cell-types (Ishikawa and Barber, 2008) in response to the activation of pat the sensors cGAS and IFI16. (Cerboni *et al.*, 2017). Following activation, STING recruits various signaling cofactors to activate IRF3 and NF-kB, thereby inducing the expression of its target genes, which include additional RFs and pro-inflammatory cytokines/chemokines. Here we show that expression of STING remains at high levels during the first 8 months of treatment, despite the significant decrease in the expression of the other RFs, including IFI16 that is one of the main drivers for STING activity. Consistently, STING levels were positively associated with the CD4+ T cell

count restoration as well as the CD4/CD8 ratio increase observed in treated patients.

The delayed decay of STING levels compared to IFI16 levels is consistent with the post-transcriptional regulation of this protein and with previous observations showing that depletion of IFI16 did not alter expression of cGAS and STING (Jønsson *et al.*, 2017). Interestingly, it has been proposed that the virus itself can repress STING activity by inducing the expression of NLRX1, an antagonizing factor able to disrupt the STING-TBK1 interaction (Guo *et al.*, 2016). Furthermore, it has also been suggested that STING might potentiate viral replication due to its ability to enhance the NF-kB pathway (Kwon *et al.*, 1998), which is crucial for HIV transcription (Ishikawa *et al.*, 2009), and thus being able to modulate STING activity might represent a selective advantage for the virus.

For it design, the present study has some limitations in determining the modulation of RFs expression in response to cART treatment. First, we did not include sequencing of host or viral genes: specific RFs gene polymorphisms as well as viral mutations can influence the interplay between viral replication and immune response (Malim and Bieniasz, 2012; Guha and Ayyavoo, 2013; Carrington and Alter, 2016; Patel and Jin, 2019; (Yi *et al.*, 2013). Second, our analysis was limited to the transcription level and thus did not take in account the protein levels and activation status. Finally, for the limited number of patients included in the study, we cannot determine whether specific antiretroviral drugs are able to modulate RFs expression (Abdel-Mohsen *et al.*, 2015; Amie *et al.*, 2014).

In conclusion, our study confirms that, by controlling viral replication, antiviral treatment also modulates RFs expression. Our data indicate the decay of interferon signaling takes longer than the time necessary to achieve viral suppression, highlighting the need to monitor immune activation and inflammation also during cART treatment.

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Conflicts of interest

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